HHS Public Access

Author manuscript

J Virol Methods. Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:

J Virol Methods. 2015 March ; 213: 157–163. doi:10.1016/j.jviromet.2014.12.001.

Molecular Genotyping and Quantitation Assay for Rotavirus Surveillance

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Abstract

Rotavirus genotyping is useful for surveillance purposes especially in areas where rotavirus vaccination has been or will be implemented. RT-PCR based molecular methods have been applied widely, but quantitative assays targeting a broad spectrum of genotypes have not been developed. Three real time RT-PCR panels were designed to identify G1, G2, G9, G12 (Panel GI), G3, G4, G8, G10 (Panel GII), and P[4], P[6], P[8], P[10], P[11] (Panel P), respectively. An assay targeting NSP3 was included in both G Panels as an internal control. The cognate assays were also formulated as one RT-PCR-Luminex panel for simultaneous detection of all the genotypes listed above plus P[9]. The assays were evaluated with various rotavirus isolates and 89 clinical samples from Virginia, Bangladesh and Tanzania, and exhibited 95% (81/85) sensitivity compared with the

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Competing interests

None declared.

Ethical approval

All studies were approved by the University of Virginia, International Centre for Diarrhoeal Disease Research, Bangladesh and Kilimanjaro Christian Medical Centre institutional review boards.

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conventional RT-PCR-Gel-electrophoresis method, and 100% concordance with sequencing. Real time assays identified a significantly higher rate of mixed genotypes in Bangladeshi samples than the conventional gel-electrophoresis-based RT-PCR assay (32.5% vs. 12.5%, P <0.05). In these mixed infections, the relative abundance of the rotavirus types could be estimated by Cq values. These typing assays detect and discriminate a broad range of G/P types circulating in different geographic regions with high sensitivity and specificity and can be used for rotavirus surveillance.

Keywords

rotavirus; diarrhea; genotyping; multiplex real time RT-PCR

1. Intoduction

Rotavirus is one of the most common causes of diarrheal disease in young children globally and leads to two million hospitalizations and more than a half million deaths every year (Parashar et al., 2009; Parashar et al., 2003). Rotavirus vaccine has been recommended by WHO for all national immunization programs (Babji & Kang, 2012; World Health Organization, 2013). Rotaviruses belong to the *Reoviridae* family and are classified into G- and P-types based on sequence or antibody reactivity to two outer viral proteins, VP7 and VP4, respectively. To date, >70 different G-type and P-type combinations have been identified (Matthijnssens et al., 2011). The G/P type of rotaviruses can fluctuate both temporally and geographically. Although cross-protection occurs with rotavirus vaccines, the extent and durability of this protection is unclear, thus uncommon strains may become prevalent or new strains may emerge under vaccine pressure (Assis et al., 2013; Gurgel, Correia, and Cuevas, 2008; Hull et al., 2011; Kirkwood et al., 2009; Matthijnssens et al., 2009; Zeller et al., 2010). Pre-rotavirus vaccine surveillance reports from 1996 to 2007 (Banyai et al., 2012) provided a comprehensive landscape of rotavirus strain distribution worldwide. Prospective longitudinal surveillance post-rotavirus vaccine has been called for using robust genotyping technologies (Dennehy, 2013; Gentsch, Parashar, and Glass, 2009b).

Multiplex RT-PCR followed by gel-electrophoresis discrimination based on amplicon length has been the primary rotavirus genotyping method (Gentsch et al., 1992; Gouvea et al., 1990). Among 281 rotavirus typing studies within 12 years (Banyai et al., 2012), nearly all of the studies used RT-PCR, with 30% in combination with sequencing. Other methods used have included southern blot, northern blot, reverse line blot hybridization, PCR-ELISA, and RFLP. Probe-based real time PCR may offer more sensitive and specific detection and avoids post-amplicon manipulation and potential risk of contamination. Many one step singleplex real time RT-PCR assays have been designed for a variety of targets for rotavirus detection, such as VP6, NSP3, NSP4, VP2, but real time RT-PCR platform has not been adapted widely for rotavirus genotyping. Recently, Kottaridi et al developed two panels of real time RT-PCR assays for detection of G1, G2, G3, G4, G9, P[4] and P[8] and showed good agreement with the conventional PCR assays but a two step was used and the selection of types was limited (Kottaridi et al., 2012).

In this work, three panels of 5-plex internally controlled one step real time PCR reactions were developed for identification and quantitation of G1-4, G8, G9, G10, G12 and P[4], P[6], P[8], P[10], P[11]. Alternately a 15-plex RT-PCR-Luminex assay was developed for identification of the same genotypes plus P[9]. These assays were evaluated with clinical specimens from three different regions of the world.

2. Materials and methods

2.1. Specimens

Representative rotavirus isolates were selected for evaluating analytical performance, including Wa (G1P[8]), DS-1 (G2P[4]), AU-1 (G3P[9]), ST3 (G4P[6]), 69M (G8P[10]), 116E (G9P[11]), I-321 (G10P[11]), L26 (G12P[4]). Fecal samples tested previously positive for rotavirus by ELISA were provided from studies at the International Centre for Diarrhoeal Disease Research, Bangladesh, Kilimanjaro Christian Medical Centre, Tanzania, and Division of Consolidated Laboratory Services, Virginia. Bangladeshi samples were selected from a birth cohort study (2008 to 2009) in the Mirpur region of Dhaka (Mondal et al., 2012). Tanzanian samples were collected from inpatients with diarrhea from Kilimanjaro Christian Medical Centre and referral hospitals in Moshi from February 2008 to June 2009. More than 70% of the rotavirus positive samples were from children under age five. Virginia specimens were rotavirus positive diarrheal specimens collected during routine outbreak investigations (from February to April, 2011) by Division of Consolidated Laboratory Services, Virginia. All studies were approved by the University of Virginia, International Centre for Diarrhoeal Disease Research, Bangladesh and Kilimanjaro Christian Medical Centre institutional review boards.

2.2. RNA extraction

Nucleic acid was extracted from fecal samples using the QuickGene RNA tissue kit SII (Fujifilm, Tokyo, Japan) as described previously (Liu et al., 2011).

2.3. Multiplex one step real time RT-PCR

Genotype specific primers and probes were designed in the variable regions of VP7 and VP4 and adapted or modified from published assays wherever feasible (Aladin et al., 2010; Gentsch et al., 1992; Gouvea et al., 1990; Iturriza-Gomara, Kang, and Gray, 2004) (Table 1). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and Biosearch Technologies (Novato, CA). Real time RT-PCR was performed with AgPath-ID RT-PCR kit (Life Technologies, Carlsbad, CA) with a CFX system (BioRad, Hercules, CA). Three panels were formulated as Panel GI, including G1, G2, G9, and G12; Panel GII, including G3, G4, G8, G10; Panel P, including P[4], P[6], P[8], P[10], P[11]. An internal control assay targeting NSP3 was incorporated in both G panels with final primer and probe concentration at 200 nM and 100 nM, respectively (Zeng et al., 2008). The samples were denatured by incubation at 95°C for 5 min then on ice prior to mixing with the one step real time RT-PCR reagents. The cycling condition included reverse transcription at 45°C for 20 min, denaturation at 95°C for 10 min, 45 cycles of 95°C for 15 sec and 55°C for 1 min.

2.4. RT-PCR-Luminex assay

RT-PCR-Luminex assays were performed with QIAamp One Step RT-PCR kit (Qiagen, Valencia, CA) followed by luminex detection with a BioPlex system (BioRad) (Liu et al., 2011). Final concentrations of biotinylated and non-biotinylated primers were 300 nM and 200 nM, respectively, for all the genotype specific primer assays, except 150 nM and 100 nM for NSP3. Carboxylate microspheres were labeled with oligonucleotide probes with amino modifier, and luminex detection was performed as described previously (Liu et al., 2011). The cutoff set for positivity was two-fold Median Fluorescence Intensity above background (nuclease free water).

2.5. Amplicon sequencing

PCR amplicon was generated with consensus primers for VP7 (Beg9 and End9) and VP4 (con2 and con3) described previously (Gentsch et al., 1992; Gouvea et al., 1990), and sequenced by GENEWIZ (South Plainfield, NJ). Con2 and con3 for VP4 were modified slightly, forward primer 5'-TGGCTTCRCTCATTTATAGACA-3', reverse primer 5'-ATTTCNGACCATTTATAWCC-3'.

2.6. Generation of RNA transcripts

Consensus VP7 or VP4 amplicon from a positive sample for each genotype was cloned, amplified and in vitro transcribed according to the previous protocol (Liu et al., 2011), with T7 RNA polymerase and SP6 RNA polymerase, respectively. The two RNA transcript products were mixed in equal molar concentration measured with Nanodrop (Bio-Rad) in 50 mM Tris, pH 8.0, 1mM EDTA, 100 mM NaCl, then denatured at 90°C for 3 min and hybridized by cooling down slowly to room temperature to generate double stranded templates. Non-denaturing 2% agarose gel electrophoresis was run to ensure the formation of double stranded RNA. For analytical performance, double stranded RNA transcripts were spiked into lysis buffer during extraction of fecal samples from healthy donors.

2.7. Statistics

Correlation was tested by regression analysis using the analysis of variance (ANOVA) tests. Mixed infection rates were compared with Chi-Square test. All *P* values were two-tailed and values of <0.05 were considered statically significant.

3. Results

3.1. Analytical performance of the real time RT-PCR assays

Linearity and limit of detection were determined using in vitro transcripts of VP7 and VP4 sequences corresponding to the interrogated G/P-types. As shown in Table 2, Pearson coefficient varied between 0.98 and 1.00 for linearity. Limit of Detection, defined as the lowest concentration to achieve 100% detection in ten spiked samples, was 10^6 copies of in vitro transcripts per gram of stool (equivalent to 200 copies per RT-PCR reaction prior to extraction). The average quantification cycle (Cq) values at the Limit of Detection were designated as the analytical cutoff used for further data analysis. Specificity of the assays was tested with 8 rotavirus isolates, each was positive for the expected G/P genotypes, and

there were no false VP7 or VP4 detections. Furthermore, no cross-reaction was observed in clinical stool samples that were positive for adenovirus (n = 8), astrovirus (n = 3), norovirus GII (n = 4), diarrheagenic *E. coli* (EAEC, EPEC, ETEC, n = 12), *Campylobacter* (n = 3), *Cryptosporidium* (n = 3), *Giardia lamblia* (n = 6).

3.2. Correlation of real time RT-PCR results with conventional genotyping results on Bangladeshi samples

Forty Bangladeshi samples were selected to evaluate the real time assays because they were genotyped previously with a conventional gel-electrophoresis based RT-PCR method (Gentsch et al., 1992; Gouvea et al., 1990). Ninety-five percent of conventional results were confirmed by real time RT-PCR (Table 3). The four unconfirmed results included two samples that were genotyped as G9 previously but were G12 with real time RT-PCR and sequencing, one sample that was genotyped as P[8] previously but non-typeable with the real time assay and failed to generate amplicon with consensus VP4 primers, and one sample that was identified as a mixed P type previously but genotyped as single P[8] by real time RT-PCR and sequencing. In addition, the real time assays detected mixed G-types and P-types in 30% and 25% of the samples, versus 10% and 7.5% by conventional methodology, respectively ($P < 0.05$). Overall multiple G or P types were identified in 33% of the samples. For mixed G or P type infections, the dominant type was evaluated by qPCR Cq (since efficiency was similar between the assays, effectively the dominant type was that with the lowest Cq). Fourteen samples were subjected to amplicon sequencing with consensus VP7 and VP4 primers and revealed 100% concordance with the dominant types (labeled with asterisks in Table 3) identified by real time RT-PCR. The minority types in these samples were amplified with one genotype specific primer combined with the corresponding consensus VP7/VP4 primer, followed by sequencing. All results were confirmed except for two samples where the amount of amplification product was too low to produce reliable sequencing data.

3.3. Correlation of real time RT-PCR results with sequencing on Tanzanian and Virginian samples

Samples from Tanzania and Virginia were selected randomly without previous genotyping information. Amplicons were generated with consensus primers for VP7 and VP4 and were sequenced as the gold-standard (Table 3). The dominant genotypes detected with real time RT-PCR assays were 100% consistent with sequencing results on 49 Tanzanian and Virginian samples. One of 11 Virginia samples had mixed G-types and 3 had mixed P-types. G12P[8] was found to be the predominant genotype. Among 38 rotavirus positive samples from Tanzania, 50% were G1P[8], 11% G12P[6], 8% G1P[6], 8% G8P[6], 5% G2P[4], 5% G9P[8], 5% G3 (P-type non typeable), 3% G12P[8], and 5% P[8] with mixed G1 and G3.

3.4. Quantitative interpretation of the genotyping results

Quantitation was used to determine the G and P type combination in mixed infections. The copy number of each genotype in a sample was calculated based on the linear regression derived from the linearity experiment. Then the relative abundance of each G or P genotype in a mixed infection could be plotted as exampled in Figure 1. In this sample, the

predominant type would most likely be G9P[8] followed by G9P[4], while the minority types could presumptively be G2P[6] and G2P[4], or some other combinations. In addition, the Cq value of the predominant type in each sample was plotted against NSP3 Cq value in the corresponding sample (Figure 2). A tight linear correlation was obtained with the set of samples tested ($n = 89$; $R^2 = 0.877$, $P < 0.001$).

3.5. Results of Luminex based RT-PCR assay

RT-PCR-Luminex showed 87% concordance with real time RT-PCR results (Table 3). Discrepancies were exclusively due to samples with low viral load, usually minority types that were not identified by RT-PCR-Luminex assay. Receiver Operating Characteristic analysis showed that RT-PCR-Luminex often lost detection for the genotypes with a Cq value after 35.2.

4. Discussion

Rotavirus has a high propensity towards reassortment due to its segmented genome. This feature makes drug and vaccine development challenging and also requires inclusive assay designs (Ghosh et al., 2012; Rahman et al., 2005). For this purpose multiplex RT-PCR panels were developed and evaluated in both real time and Luminex platforms for specific identification of various G/P types of rotavirus (Table 1). These were validated with 8 representative rotavirus isolates and 89 rotavirus samples from three continents, which possessed divergent nucleic acid sequences (data not shown). The results confirmed conventional gel electrophoresis based RT-PCR typing methods and sequencing, and this assay could be used for future rotavirus surveillance.

A few findings were notable. The Tanzanian samples revealed G1P[8] to be common, consistent with a recent report (Moyo et al., 2014), but contrasting to a study showing G9P[8] to be the major genotype in Tanzania (Moyo et al., 2007). Whether this reflects temporal fluctuation or geographic differences needs further study, and emphasizes the need for continuous surveillance of rotavirus genotypes to understand natural fluctuation versus vaccine pressure. In Virginia the majority of infections were G12. G12 has been reported as an emerging genotype in the United States (Freeman et al., 2009; O'Ryan, 2009; Payne et al., 2008), but is usually rare from the prevaccine era (Gentsch et al., 2009a). This also highlights the rationale for post-vaccine genotypic surveillance. Next, a higher 212 percentage of mixed infection was detected than with conventional methods, especially in Bangladeshi samples. This is a region of high rotavirus transmission and reassortment (Unicomb et al., 1999), and mixed genotype infection has certainly been reported elsewhere (Fischer et al., 2005; Fischer et al., 2003; Nielsen et al., 2005). In addition, these real-time assays are highly sensitive and RT-PCR followed by gel electrophoresis and Sanger sequencing has limited resolution. Quantitation was found useful to determine the dominant type in each sample, and this type was 100% consistently detected with sequencing and 92% (72/78) with RT-PCR-Gel-electrophoresis. Quantitation can also be used to match the G type with the likely corresponding P type (Figure 1). The clinical importance of mixed infections, or of dominant types, needs further investigation. Obviously mixed infection

could complicate an assessment of vaccine efficacy or of diarrhea etiology (Linhares et al., 2006).

The limitation of these and most amplification-based assays is that limited genotypes are interrogated even though new types are emerging. Untypeable strains have been reported at an average rate of 30% in African countries when RT-PCR-gel electrophoresis method was used (Mwenda et al., 2010). To get a full picture of strain distribution requires either updating the assays and targets, or developing new sequencing-based strategies without the need for a priori sequence information. Whole genome sequencing analysis has recently been implemented to characterize circulating strains in certain regions. It can be envisioned these PCR panels be used as a quick screening tool to determine if such analysis is needed. Since an assay targeting a highly conserved region of NSP3 was included as internal control, and since there exists a tight correlation between NSP3 and genotype specific signals (the dominant types), it is possible to infer if any genotype is escaping detection by comparing viral burdens deduced from Cq values of the dominant type and NSP3, respectively.

Regarding the use of the real time versus Luminex platforms, reagent cost is similar and each has tradeoffs. Both have lower instrument and bioinformatics requirements compared with sequencing. Real time is limited by the availability of fluorophores and the instrument's capability. RT-PCR-Luminex is limited by post amplification manipulation, but enables single reaction to detect all the genotypes simultaneously. For the use in field studies the real time PCR assays is favored, since this procedure is faster and less prone to contamination. The original design was intended for a leading panel (GI) to detect G1, G2, G9 and G12, the major strains in Bangladesh. A simplified screening process for rotavirus strain surveillance could run panel GI first, then run panel GII only when the results for panel GI were negative or on any sample having high rotavirus burden indicated by NSP3 signal but low burden by genotypes in panel GI (Figure 2). It is possible that Tanzania has a different rotavirus strain profile (with G1 being the dominant type followed by G12, G3, G8 and G9), therefore the targets between two G panels were switched and the leading panel for these types was formulated successfully (data not shown).

A limitation of this study is that convenience samples from a community-based study (Bangladesh), hospitalized cohorts (Tanzania), and outbreaks (Virginia) were examined, thus the inferences that can be drawn regarding strain distribution in these geographies and settings are limited. In summary, these assays detect both predominant and relatively uncommon genotypes simultaneously, and can serve as a fast screening tool to help understand potential changes in epidemiology post rotavirus vaccine.

Acknowledgments

Funding

This study was supported by Bill and Melinda Gates Foundation (PROVIDE, OPP1017093) and NIH (RO1 AI043596).

Abbreviation

RT-PCR	reverse transcription polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
Cq	quantification cycle

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Highlights

- We developed multiplex real time/Luminex panels for rotavirus genotyping.
- Analytical performance was validated with various rotavirus isolates.
- Accuracy was 95-100% compared with conventional RT-PCR methods.
- The relative abundance of the rotavirus types could be estimated with Cq values.
- These rotavirus genotyping assays can be used for rotavirus surveillance.

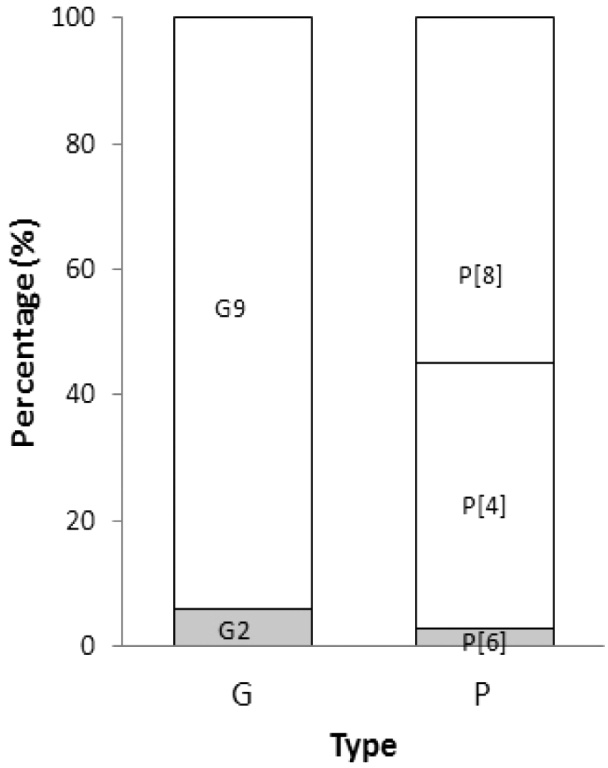

Figure 1.

Relative abundance in a mixed infection. The relative abundance was normalized to NSP3 signal, as the measure of total rotavirus burden in a sample. Based on the fraction of each G- and P-type in this example, G2P[6], G2P[4], G9P[4], and G9P[8] could presumptively be assigned to this particular sample with the latter as the dominant type.

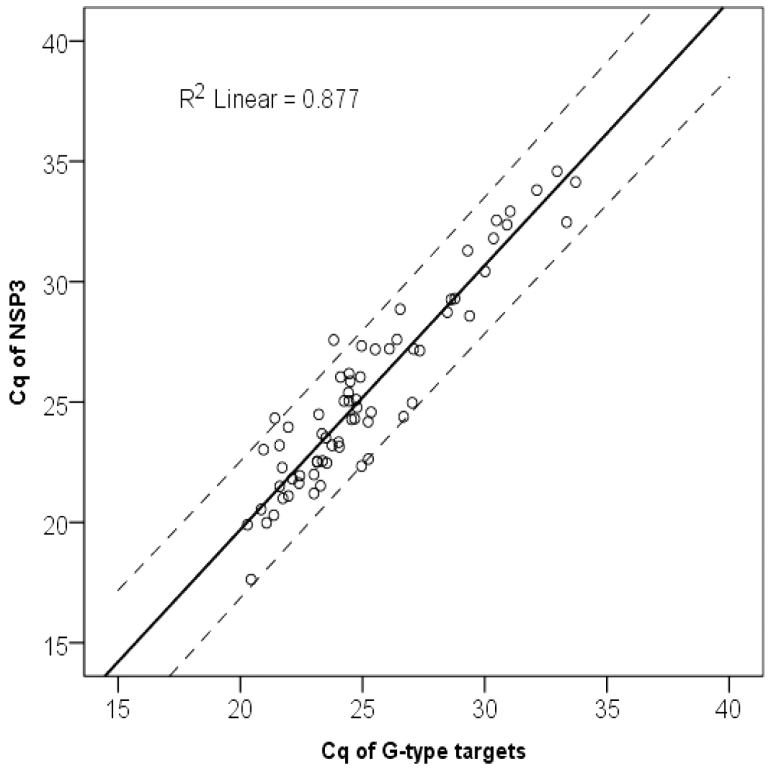

Figure 2.

Correlation of NSP3 Cq values with G-type specific targets. The solid line shows the linear regression (Pearson coefficient $R^2 = 0.877$; $P < 0.001$), with the broken lines the 95% confidence interval. All the samples tested positioned above the lower bound of 95% confidence interval. However assuming a sample had a Cq at 25 for NSP3 and 33 for G1 as the dominant type tested, a G type that was not interrogated by panel GI and GII might be present in this sample.

Table 1

Primer and probe sequences for real time RT-PCR assays. For the cognate RT-PCR-Luminex assays, the forward primers were biotinylated (IDT, label with 5'-BioSg) and the probes were labeled with amino modifier (IDT, 5'-AmMC12).

Target	Genotype	Sequence	Concentration in real time RT-PCR reaction (nM)	Fluorophore	Reference
VP7 (panel I)	G1	F: ACWTACCAATAACAGGATCAATGGA	500		This study
		R: AATGAITCRTTCCAKTCACCATCA	500		This study
		P: TCCAACWGAAGCAAGTACTCAAA	250	Quasar 705	This study
	G2	F: GCATCIGARTTAGCAGRTCTTA	300		This study
		R: TACCGTGCAGTCIGTTCCCAT	300		This study
		P: CCATAGGATTGCACAGCCA	150	HEX	This study
	G9	F: CCATAAACTTGATGTGACTAYAAATAC CCATAAACTTGATGTGACTACGAGTAC	300 300		(Gouvea et al, 1990; Iturriza-Gomara et al, 2004)
		R: TGYAGTAGTTGGATCYGCTGTA	300		This study
		P: TCTAACACATCTGAGCCACC	150	FAM	This study
	G12	F: TGGTTATGTAATCCGATGGACG	600		(Aladin et al, 2010)
		R: AATGTTGYGACGTCGGTTGT	600		This study
		P: CCCATTGATATCCATTTATT	300	Texas Red	This study
VP7 (panel II)	G3	F: ACGAACTCAACRCGAGAGG	400		(Iturriza-Gomara et al, 2004)
		R: GTTGCTGCTTCAGTTGGGTAATA	400		This study
		P: TTCCTRACTTCGACTTTATGTTT	250	FAM	This study
	G4	F: GGGTCGATGGAAAATTCT	400		This study
		R: ATCAGAAGCTCCAACTCAAA	400		This study
		P: ATAAACTGAACCTGTCGGCC	200	Texas Red	This study
	G8	F: CCAGTTGGCCAYCCTTTTGT	500		This study
		R: TTGTCACACCATTTGTRAATTC TTGTCACACCATTCGTAAACTC	500 500		(Aladin et al, 2010; Iturriza-Gomara et al, 2004)
		P: TTCCAYGAACTATCWGCTAT	300	HEX	This study
	G10	F: GACGAAGCAAAYAAATGGATAGC	400		This study
		R: TGACATCCTATYCCTAGYGTTT	400		This study
		P: CATGATTGTCCCATYGCT	300	Quasar 705	This study
VP4	P[4]	F: TCCGCAGTAYTYGAACTATCAG	200		This study
		R: GACGGACTYTAACCTCTAAYAATAG	200		(Gentsch et al, 1992)
		P: TTCATGGTGAAACACCAAGAG	100	Texas Red	This study
	P[6]	F: TTAATCCCGGACCRTTTGC	300		This study
		R: ACAACTTGTTGATTAGTTGGATTC	300		(Gentsch et al, 1992)
		P: TCACTTCCCCATGACTCCAA	150	HEX	This study
	P[8]	F: TGGRTTRACNTGCGGTTCAA	200		(Iturriza-Gomara et al, 2004)
		R: GACGGTCCTTATCAGCCTACTAC	200		This study

Target	Genotype	Sequence	Concentration in real time RT-PCR reaction (nM)	Fluorophore	Reference
		P: AATAGTGACTTTTGGACTGCAG	100	FAM	This study
	P[10]	F: CTGACCACCGTGCTTCATTA	200		This study
		R: TGAAAACCACRTCATCAGGAA	200		This study
		P: TATCAGAGCCAAAACTCTATGG	100	Quasar 670	This study
	P[11]	F: GTTGCGAATCTGGTATRACG	500		This study
		R: AAGGTGATTIGAGRGTTGGAA	500		This study
		P: TGCAGTGATCAATCTAAATGC	250	Quasar 705	This study
	P[9]*	F: TGAGACMTGYAATTGGACATTTTG	-		(Iturriza-Gomara et al, 2004)
		R: GAAGGRAAAGTTGCTGAAGGTA	-		This study
		P: AAGRCAATACGTATTAGATGG	-		This study

*Only included in RT-PCR-Luminex panel.

Table 2

Analytical performance of real time RT-PCR assays. Limit of detection was defined as the lowest concentration at which the target could be detected in all 10 spiked samples. The corresponding average Cq at the LoD was used as analytical cut-off.

Genotype	Calculated PCR efficiency	Linearity, R^2	Limit of Detection	
			Copy No./reaction prior to extraction	Corresponding Cq
G1	94.6%	0.987	200	38
G2	90.5%	0.999	200	36
G3	93.4%	0.998	200	36
G4	94.5%	0.992	200	37
G8	94.2%	0.996	200	36
G9	90.9%	0.980	200	36
G10	87.3%	0.998	200	38
G12	99.1%	0.999	200	37
P[4]	86.8%	0.981	200	37
P[6]	98.2%	0.991	200	36
P[8]	83.9%	0.990	200	36
P[10]	98.2%	1.000	200	36
P[11]	98.7%	0.991	200	36

Table 3

Comparison of real time RT-PCR and RT-PCR Luminex results with conventional rotavirus genotyping methods or sequencing. Any genotype with relative abundance less than 2% in a mixed infection was not included.

Sample source	Comparator method**	Real Time RT-PCR		RT-PCR Luminex	
		Type	Count	Type	Count
Bangladesh	G1, P[8]	G1, P[8]	3	G1, P[8]	3
	G1, P[8]	(G1*, G12), (P[6], P[8]*)	1	(G1*, G12), P[8]	1
	G1, P[8]	(G1*, G2, G9), P[8]	1	G1, P[8]	1
	G2, P[4]	G2, P[4]	9	G2, P[4]	9
	G2, P[4]	G2, (P[4]*, P[8])	1	G2, P[4]	1
	G2, P[4]	(G2, G9*), (P[4]*, P[8])	1#	G9, (P[4]*, P[8])	1
	G2, P[6]	(G2, G9*), (P[4], P[6]*)	1#	G9, P[6]	1
	G9, P[6]	G9, P[6]	1	G9, P[6]	1
	G9, P[6]	G12, P[6]	2#, ¶	G12, P[6]	2
	G9, P[8]	G9, P[8]	5	G9, P[8]	5
	G9, P[8]	(G1, G9*)	1¶	G9	1
	G9, P[8]	(G2, G9*), (P[4], P[6], P[8]*)	1	G9, P[8]	1
	G12, P[6]	G12, P[6]	6	G12, P[6]; G12	5; 1
	G12, P[6]	(G1, G2, G9, G12*), (P[6]*, P[8])	1	(G2, G12*), (P[6]*, P[8])	1
	G12, P[8]	G12, P[8]	1	G12, P[8]	1
	(G2, G9), P[4]	(G2, G9*), (P[4]*, P[8])	1	(G2, G9*), (P[4]*, P[8])	1
	G9, (P[6], P[8])	(G2, G9*), (P[6], P[8]*)	1	G9, P[8]	1
	(G9, G12), P[8]	(G9*, G12), (P[6], P[8]*)	1	(G9*, G12), P[8]	1
	(G2, G9), (P[4], P[8])	(G2, G9*), (P[4], P[8]*)	1	G9, (P[4], P[8]*)	1
	Mixed G and P$	(G1, G2, G9*), P[8]	1¶	G9, P[8]	1
Virginia	G2, P[4]	G2, (P[4]*, P[8])	3	G2, P[4]; G2, (P[4]*, P[8])	1; 2
	G12, P[8]	G12, P[8]	7	G12, P[8]	7
	G12, P[8]	(G2, G12*), P[8]	1	(G2, G12*), P[8]	1
Tanzania	G1, P[8]	G1, P[8]	19	G1, P[8]	19
	G1, P[8]	(G1*, G3), P[8]	2	G1, P[8]	2
	G1, P[6]	G1, P[6]	3	G1, P[6]	3
	G2, P[4]	G2, P[4]	2	G2, P[4]	2
	G3	G3	2	G3	0
	G8, P[6]	G8, P[6]	3	G8, P[6]; P[6]	2, 1
	G9, P[8]	G9, P[8]	2	G9, P[8]	2
	G12, P[6]	G12, P[6]	4	G12, P[6]	4
	G12, P[8]	G12, P[8]	1	G12, P[8]	1

[*] dominant genotype by quantity in the mixed infection.

[**] The comparator method for Bangladesh samples was RT-PCR followed by gel electrophoresis detection; for Virginian and Tanzanian samples, it was amplicon sequencing.

[#] Real time results were confirmed by sequencing.

[$] The specific types could not be identified with conventional methods.

[¶] unconfirmed cases